Bacterial Variation since Pasteur*

Rummaging in the attic: antiquarian ideas of transmissible heredity, 1880–1940

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I began my scientific work on neurosporas in 1942 (at the age of 17 at Columbia University in New York). Avery, Macleod, and McCarty's 1944 report on the chemistry of the pneumococcal transforming factor—as DNA!—irrevocably oriented my scientific career to questions of the genetic structure of bacteria. Part of my preparation for experimental study in that field was a review of the literature: I was guided particularly by René Dubos' *The Bacterial Cell,* which truly is an encyclopedic bellwether of the modern era, as well as a guide to much of the older literature. In 1946, I moved to the laboratory of E. L. Tatum at Yale University and successfully consummated the first experiments on crossing *Escherichia coli* K-12, begun at Columbia, in time for the Cold Spring Harbor Symposium in July 1946. That occasioned my first introduction to André Lwoff and Jacques Monod and my unbroken connection with the Pasteur Institute.

So much of the early groping towards a bacterial genetics was flawed by both intellectual confusion and messy experimental reporting. Work of this kind is worse than useless for several reasons: it creates an aura of disreputability that repels younger investigators, and it establishes spurious theoretical paradigms. In addition, what incentive is there to clean up messy work? If it is disconfirmed, that is a social service that tends to glean little credit for the labor invested. If by some chance it should be confirmed, it will merely exalt the reputation of the first claimant, and for limited deserts. No wonder that so many early observations were simply allowed to molder.

On the other hand, it is exciting to see some of the more clear-sighted early explorations, like those of Pasteur, Theobald Smith, and MacFarlane Burnet, which have given us an enduring legacy. Not that they have been, or could have been, always on the right side of every argument: we can even glide over Pasteur's attachment to vitalism, when he gave us so much of the evidence needed to overthrow that philosophy!

The lessons to be learned from excavations of the scientific literature, besides giving due historical tribute, include a series of clinical case studies in the pathology of scientific development. In addition, there have been and doubtless will continue to be more "sleepers," forgotten ideas and experimental observations that deserve reexamination today for fresh interpretation, sometimes with quite exciting consequences.

My main themes will be:

- Attenuation, exemplified by Pasteur's attenuation of anthrax with high-temperature cultivation in 1881, and its recent corroboration as plasmid loss.
- Paragglutination: exchange of "receptors" between bacteria in mixed culture and infections.
- Lysogenic conversion—and some possible anticipations: "filtrate effects."
- Griffith's discovery of transformation in pneumococci, 1928.
- Unfinished business (the Weil-Felix reaction): antigenic cross-reactions of rickettsiae with *Proteus* X strains.

Attenuation

In 1881, Pasteur developed a protective vaccine for

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anthrax by growing his cultures at marginal growth temperatures of ~43°C. This initiated the prevalent empirical practice of "attenuating" virulent pathogens, be they bacteria or viruses, by cultivation in adverse environments or unconventional hosts. Before the 1940s, little sophisticated analysis was devoted to the genetic mechanisms that might be involved; indeed, few would have troubled to think about a genotype as distinct from the expressed phenotype. Theobald Smith apart, few of the earlier workers even thought of a culture as a population of possibly disparate cells. Pasteur devoted little technique or trouble even to achieving pure culture. Koch, who was its zealous proponent, blamed most observations of bacterial variation on contamination, and with F. Cohn upheld the "monomorphism" (genetic invariance) of bacteria.

As genetic approaches began to emerge in bacteriology, the only systematic study of variation of virulence factors in aging bacterial cultures by Werner Braun supported a rather banal interpretation of selective advantage of "rough variants" in such cultures. Nevertheless, the idea of symbiotic particles, later called plasmids, playing a role in microbial heredity was so intriguing that in 1949 I gambled by using Pasteur's expression "attenuation" for plasmid loss. This has lately been wonderfully vindicated: avirulent cultures of anthrax lack a critical virulence plasmid. Ezzell and colleagues point out, ironically, that Pasteur's nonchalance about pure clone cultures, so much criticized by Koch, was the key to the success of his anthrax vaccine, which was a mixture of plasmid-deprived and still toxic (and immunogenic) plasmid-positive cells. The removal of plasmids by "pasteurization" of bacterial cultures has played an equally incisive role in the analysis of plasmid function for the production of toxin crystals in *Bacillus thuringiensis*. Many other virulence functions are also attributed to plasmids in contemporary microbiology.

The loss of "killer plasmids" by heating is paralleled by the removal of kappa particles of paramecia by heating and has a close analog in the removal of chloroplasts with streptomycin. Before long, I encountered the F factor as a plasmid in my own work with *E. coli* K-12 and was delighted eventually to find that it could be attenuated by cultivation in the presence of acriflavine but not heat. Boris Ephrussi's success in inducing cytoplasmic "petites colonies" mutations in yeast was the immediate inspiration for that effort. Many other substances, mainly DNA-binding molecules, will remove plasmids from bacteria.

In my 1949 review, I mentioned two other reports of attenuation that might deserve further scrutiny—they still do. *Agrobacter tumefaciens* cultivated on glycine loses its plant tumor-inducing competence, a trait now well known to be dependent on a large plasmid. Mucoid group C hemolytic streptococci lose their mucoid capsules when grown on acetate-free media. I am not aware of any follow-up to those reports in light of current knowledge.

Paragglutination

The possibility of the transfer of traits, particularly antigenic markers, from one bacterial species to another is a recurrent theme in the bacteriological literature between 1890 and 1930. The theoretical basis for these speculations was often the "absorption of heterologous receptors," exemplified in the passive sensitization of erythrocytes, bacteria, or collodion particles.

For example, in 1909, Kuhn and Woithe noted that *E. coli* from a dysentery case reacted with *Shigella flexneri* Y antiserum, cross-reacting with dysentery organisms later isolated from the same patient. Speculations of biological transfer led to experiments in which *E. coli* cultures were grown together with the shigellae and acquired a cross-reaction with the shigellae. Aware of these reports, Eugene and Elisabeth Wollman in 1925 recorded a similar observation with *E. coli* and *Salmonella paratyphi* B. They devoted their attention thereafter to studies of lysogenicity at the Pasteur Institute. They coordinated this hypothetical transfer of receptors with the phenomenon of bacteriophagy and suggested the term "paraheredity." This brilliant theoretical anticipation of lysogenic conversion was, unfortunately, not matched by further experimental inquiry.

Retrospective efforts to explain paragglutination should distinguish in vitro experiments from the natural occurrence of exceptional cross-reactions, e.g., of *E. coli* strains with more or less complete *Salmonella* or *Shigella* antigens. The latter reactions certainly occur, and today we would not resist interpreting them as instances of genetic recombination. In addition, many organisms share a cosmopolitan rough antigen, giving rise to secondary cross-reactions of bacteria tested as rough variants. Such variants may also be selected during laboratory culture and by encounters with antigen-specific bactericides such as phages, colicins, and complement. There has been great skepticism about the invocation of a "transfer of receptors", viz., genetic exchange, especially for want of crisp experiments with well-defined markers. Many of the earlier experiments suffer from an uncritical faith in the specificity of immunological reagents, as well as from a confusion of phenotype and genotype. Both errors of dogma litter the history of immunology as well as bacteriology.

Nevertheless, in hindsight, some of these antique experiments plausibly were a window onto real phenomena, perhaps of conjugal transfer of bacterial chromosomes or plasmids, perhaps of lysogenic conversion, that is, the dual role of a gene in the economy of a virus and of its host cell.

Lysogenic Conversion

An early experiment that may have anticipated lysogenic conversion was reported by Sanfelice—a name we associate with the etiology of cryptococcosis—in 1893. In experiments conducted barely a year after the demonstration of the toxin, nontoxic "pseudotetanus" clostridia were inoculated in filtrates of the anaerobic sporeforming *Clostridium tetani* and

A Fruitful Search in the Attic of Ideas

Joshua Lederberg pioneered microbial genetics. For example, by showing that genetic information could be passed from one bacterium to another, his work was among the first manipulations of any organism's genetic material and in large part established the importance of bacteria in genetic research. For his work on genetic structure and function in microorganisms, he shared the 1958 Nobel Prize with George W. Beadle and Edward L. Tatum.

" 'Rummaging in the attic' of the biomedical literature played an important part in the development of my ideas on bacterial genetics," Lederberg notes. "While working on my doctoral thesis (in microbiology), I spent a summer at Woods Hole [Marine Biological Laboratory] and found the library to be a remarkable resource.

"The historical literature was of great interest to me when I was first wondering 'Is there a genetics of bacteria?' I examined the existing observations and bacterial systems and their credibility and found many things that informed my later work.

"After I read about schemes for serotyping in salmonella and saw how each new antigen combination was given a new species name, I had added incentive for investigating genetic recombination going on in the microbial world. Not many years later this led to the finding (with Norton Zinder) of transduction of markers by viruses as a method of genetic exchange," Lederberg explained.

After a period of study at Columbia University School of Medicine, Lederberg received his Ph.D. in microbiology at Yale. Although he has worked with many outstanding scientists, he speaks most fondly of his mentor, Francis Ryan. "He was an extraordinary, bright, generous human being, a wonderful sparring partner who offered both discipline and respect. Everybody adored him, and some of his work in mutagenesis is being 'exhumed' today."

Lederberg

Following the receipt of his doctorate, Lederberg served as professor of genetics at the University of Wisconsin and then at Stanford School of Medicine. From 1978 to 1990, he served as president of The Rockefeller University. As a university professor, he continues his research at Rockefeller in the field of transcriptional specificities in mutagenesis in bacteria.

"Even with all of its problems, science today is better off than a half-century ago," Lederberg notes. "As a newly minted Ph.D., I was facing the issue of 'could you find a job to earn a living at doing research?'

"Even though we've seen some dimming of unblinking support for scientific research and molecular biology is of course much more crowded, any of my students still has a crack at revolutionary discovery if they will but seize the day," he said.

Although public scrutiny of scientific research and standards of accountability are more stringent, perhaps more hostile than in the recent past, Lederberg doesn't see a recrudescence of scientific McCarthyism. "Yes, the screws are a little bit tighter, and people are going to look more closely at marginal research, including plagiarism as well as imputed fraud. However, anyone who exercises a modicum of common sense and integrity has no rational basis for being deterred.

"Unfortunately, social vigilance about the integrity of scientific research may create the impression that the discipline is loaded with crooks and predators," Lederberg said. "We urgently need to dispel the idea that the primary motivation of researchers is to beat their competitors. I firmly believe that idealism and the excitement of discovery are necessary parts of science."

thereby became toxicogenic, the acquired toxicity persisting for several transfers. He attributed the effect to the toxin itself: one can excuse him for not anticipating a post-Mendelian distinction between genotype and phenotype! It is easy to suggest that Sanfelice's filtrates still had some spores. I am not aware of any effort to confirm Sanfelice's findings during the century after his report; of course a precise repetition is hardly feasible without Sanfelice's identical strains. In recent work, a conversion of toxigenicity in *C. botulinum* has been authenticated. *C. tetani* may have its toxin encoded on a large plasmid.

In their pioneering study of lysogenicity in staphylococci, Burnet and Lush (1936) reported a pigment change of a *Staphylococcus albus* to *S. aureus* after exposure to a phage B—this, to me, has the "smell" of lysogenic induction. However, their investigations focused on a phage C, work that led them to the discovery of a mutation that leads a bacteriophage to lose its lysogenizing power. Referring to the insusceptibility of the lysogenic bacteria, they concluded, "According to [the] Wollmanns' hypothesis, the distinction between the two alternatives [altered genetic constitution of the bacteria or induction by the phage] would disappear, the phage being regarded as a gene re-introduced into the genetic make-up of the organism." These thoughts had a profound effect on my own thinking about virus-gene relationships and the concept of the plasmid.

Empirical corroboration of this paradigm came from Victor Freeman in his discovery of lysogenic conversion of *Corynebacterium diphtheriae* in 1951. According to his personal account:

> While carrying on investigations with phage B it was discovered that this phage had a marked activity on several avian cultures of C. diphtheriae. In view of this result it was decided to test for the possibility of

the presence of a dermal necrotic endotoxin after the method of Lazarus and Gunnarson, J. Bact. 1947, 53:705–714. The phage lysates of the avian cultures did produce a dermal factor toxic for guinea pigs. Further investigations soon revealed that the toxic substance being produced was true diphtheria toxin. In view of these findings the principal emphasis of the research project was modified to take advantage of these very pertinent observations etc. There was no practical development of a phage typing system. Several interesting patterns of specific lysis were developed through phage adaptation but owing to this instability these tests could not be utilized for typing purposes.

In 1927, Frobisher and Brown had the idea that scarlatina was caused by a virus only secondarily related to the streptococcus. They evidently did convert a "cheese strep" to an erythrogenic one with filtrates. Their work was repeated by Bingel in 1947 and taken up again by John Zabriskie 20 years after that. Finally, Ferretti's group in Oklahoma recently put the finishing touches on this as another canonical lysogenic conversion, the toxin genes having been located on the phage (plasmid).

In 1953, I was collaborating with P. R. Edwards on the serotype of the H antigens of salmonellae. He told me of the conversion of some of the O somatic types with antisera. I tried to persuade him that antibody was a very unlikely reagent for transformation and that he was just seeing another example of selection. Zinder and I had just demonstrated phage-mediated transduction. So I asked, was there possibly some phage in his antisera? He countered that there was probably dissolved antigen being taken up by the altered bacteria. Indeed there was a phage! Iseki and Sakai, in the first of many such examples, demonstrated in 1953 the modulation of somatic antigen specificity in *Salmonella* spp. by lysogenic conversion. That is, the phage genome includes determinants altering its host's development.

DNA Transformation

Fred Griffith discovered type transformation in pneumococci in 1928. This was followed by the work of Oswald Avery. The epochal paper by Avery, Macleod and McCarty followed 16 years later in 1944, in effect the discovery of DNA as a genetic agent.

Griffith was not very revealing about the inside history of his experiment; he was generally a private person. Hardly any of his papers survive. A civil servant in the British Health Service, Griffith initiated the serotypic classification of streptococci and pneumococci that we use today. That classification is invaluable in the tracing of epidemics and in planning for serotherapy. A "smooth ↔ rough" (S ↔ R) variation was often observed, usually reversible on further cultivation or inoculation into mice. Griffith was obviously much concerned about the conditions of this variation and its bounds, but he never saw mutation of one type to another.

Griffith believed that the revertible R strains contained some residue of S "antigen"; large inocula might provide enough "pabulum or stimulus" to enable the reversion from R to S. However, he was fairly careless about phenotypic versus genotypic effects—and he certainly lacked that vocabulary. His colleague Stewart Elliott suggested to me that Griffith may also have had in mind effects like those of mucins in protecting inocula during their incubation in test animals.

Griffith conducted a series of transformation experiments using homologous strains. A stable R, derived from S-1 (which we designate R-1 although it exhibits no S-1 antigen), would engender smooth virulent revertants in the mouse when inoculated with heat-killed cultures of type 1. This would be in accord with his "pabulum" hypothesis. However, he later got S-1 as well when he used live R-2 cells! This is the paradigm of the transformation experiment; in a variety of other combinations, the killed S (smooth) cells transmitted their antigenic specificity to the rough recipients, regardless of their original type.

Griffith knew he was onto something. And from this point, he was much more discerning about his terminology. Since the antigen itself is stable to heat whereas the transforming factor is destroyed by boiling, he clearly recognized the distinction between antigen and antigen-forming system (what we today call the gene): "By S substance I mean that specific protein structure of the virulent pneumococcus which enables it to manufacture a specific soluble carbohydrate."

We are at a loss trying to trace the intellectual influence behind his experiment, and such a statement, so modern in its outlook! He must have been aware of paragglutination, if only the Weil-Felix reaction. He cites other articles from the *Zentralblatt für Bakteriologie* (Seligmann, 1917) regarding the diversification of dysentery strains. But he left no tangible clues. What happened after that is, as they say, history.

Unfinished Business: *Proteus* Cross-Reaction with Rickettsiae

In 1916, Weil and Felix isolated *Proteus* strain X-19 from a patient; it agglutinated in cross-reaction with convalescent typhus sera. Other *Proteus* isolates, the XK and X2 strains, also cross-react with other rickettsiae. However, proteus is discounted as an etiological factor in rickettsial disease. Although it is generally assumed that these are merely accidental cross-reactions of polysaccharides, this coincidence seems remarkable. Rickettsiae are not believed to have a close affinity to proteus in terms of their global DNA homologies, but that is no argument against the borrowing of genes across taxonomic boundaries. The Weil-Felix test is still occasionally used for diagnosis but has been supplanted with preparations from cultured rickettsiae. It is not clear whether purified *Proteus* preparations would fare less well.

There are many cross-reactions known today of human blood group antigens with bacterial polysaccharides, e.g., with type XIV pneumococci. We can even refer to a tradition of parasites borrowing their hosts' immunospecificity. It may be unlikely, but it is too soon to exclude that the transaction can occur at the DNA level.

Untapped Lessons from History

What is the use of rummaging through the attic of forgotten experiments? Most science is done in response to immediate contemporary stimuli such as methods, provocative data, or trendy notions about what is exciting and important. Most students have an absolute aversion to historic reminders, an allergy to the dust in the library stacks. All they need is in this month's journals.

Paradoxically, it may be in the opening of new territories, more than in the cultivation of the neighboring fields, that historical retrieval may have the greatest use. There are still old treasure maps with hints of buried troves.

In my own experience, historical material motivated new experiments on several occasions. Above all, the *Salmonella* serotypes worked out in the 1920s and 1930s were a distinct inspiration to me in 1945, encouraging the search for bacterial recombination in *E. coli* and what turned out to be transduction in 1952. *Salmonella* flagellar phase variation, which dates to Andrewes (1922) and was taken up by Iino and me in 1956, proved a gold mine of new perspectives on gene regulation. Massini's 1907 *E. coli* "mutabile," $Lac^- \rightarrow Lac^+$, spawned even more illustrious progeny, nurtured at the Pasteur Institute. A century of argument on the biology of symbiosis is the patrimony of the plasmid concept. Somatic cell genetics was given a fresh start by antiquarian pathological histology on cell fusion in viral lesions.

More problematic are historic reports in which only by luck can the experimental claims be reconstructed with enough rigor to instill confidence in them. Bacterial variation, unfortunately, has more than its share of such publications. This field has also been hindered by the lack of communication between the new biology of Mendel and Darwin on the one hand and the empirical successes of "classical" medical bacteriology on the other. For the latter, population analysis was very slow in taking hold, although it should have been implicit in the Koch-Cohn paradigms of pure culture technique. Perhaps we should also be more sympathetic with the old-liners as we realize that bacteriology did have some authentic phenomena of environmentally induced change, such as plasmid attenuation, on top of the selective phenomena and phenotypic enzyme induction that occasioned so much confusion.

As we trace the history of bacterial variation, we see a fluctuating development that is by no means linear progress. The turn-of-century insights of Theobald Smith, Beijerinck, and a handful of others were supplanted by the morass of "bacterial dissociation" and cyclogeny. One may well say that knowledge can be lost as well as gained from generation to generation; stalled theories are better forgotten than taxed with the aura of disrepute and futility.

In his address to the British Association in 1884, Lord Rayleigh remarked, "By a fiction as remarkable as any to be found in law, what has once been published, even though it be in the Russian language, is usually spoken of as 'known', and it is often forgotten that the rediscovery in the library may be a more difficult and uncertain process than the first discovery in the laboratory." I am not sure that I would match my present labors with those in the laboratory, but I have inestimable help from a technology of bibliographic retrieval unknown in Rayleigh's day. That technology may assist in the union of past, present, and future. □

Suggested Reading

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